Layer-by-layer co-immobilization of soluble complement receptor 1 and heparin on islets

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Abstract

Early graft loss due to instant blood-mediated inflammatory reactions (IBMIRs) is a major obstacle of clinical islet transplantation; inhibition of blood coagulation and complement activation is necessary to inhibit IBMIRs. Here, human soluble form complement receptor 1 (sCR1) and heparin were co-immobilized onto the surfaces of islet cells. sCR1 molecules carrying thiol groups were immobilized through maleimide-poly(ethylene glycol)–phospholipids anchored in the lipid bilayers of islet cells. Heparin was immobilized on the sCR1 layer via the affinity between sCR1 and heparin, and additional layers of sCR1 and heparin were formed layer-by-layer. The sCR1 and heparin molecules in these layers maintained anti-complement activation and anti-coagulation activities, respectively. This promising method could be employed to reduce the number of islet cells required to reverse hyperglycemia and prolong graft survival in both allo- and xeno-islet transplantation.

1. Introduction

Early graft loss is the major obstacle during transplantation of clinical islets of Langerhans (islets) [1]. When islets are transplanted through the portal vein to liver tissue in a clinical setting, exposure of the islets to blood triggers a thrombotic/inflammatory reaction [2]. Instant blood-mediated inflammatory reactions (IBMIRs) have been identified as the main reason for islet loss in the early post-transplantation stage [3]. Two to three donors per recipient are generally necessary to achieve insulin dependence [4]. Several experimental approaches have been proposed to prevent IBMIRs, including systemic treatment [5-8], and although systemic administration of drugs or proteins is effective, it is usually associated with a risk of complications and side effects. Therefore, local inhibition of the coagulation cascade has also been attempted [9-11].

We have also made various attempts [12-16] to suppress IBMIRs using amphiphilic polymers such as poly(ethylene glycol)–phospholipid conjugates (PEG-lipids) and poly(vinyl alcohol) carrying long alkyl side chains. These molecules spontaneously incorporate into the cell membrane via hydrophobic interactions between the alkyl chains and the lipid bilayer of the cell membrane. In addition, various bioactive substances such as urokinase, thrombomodulin, and human soluble form complement receptor 1 (sCR1) have been immobilized on islets through the bilayer [17-20]; sCR1 is a potent inhibitor of both the classical and alternative complement activation pathways [21-23], and urokinase and thrombomodulin can inhibit formation of blood clots on the islets. In this study, we attempted to engineer both anti-thrombogenic and anti-complement properties in the islet cells by co-immobilizing sCR1 and heparin layer-by-layer, and the effects of these layers were examined *in vitro*.

2. Materials and Methods

2.1 Purification and thiolation of sCR1

Human sCR1 was prepared using Chinese hamster ovary (CHO) cells expressing sCR1 (CRL-10052[TM], ATCC; Manassas, VA, USA) as described previously [24]. Briefly, CHO cells were maintained in α-MEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 500 nM methotrexate (Sigma-Aldrich, St. Louis, MO, USA) to form a confluent cell monolayer. Medium was then changed to ASF104 (Ajinomoto Co. Inc., Tokyo, Japan) supplemented with 500 nM methotrexate and cultured for an additional 2 days. sCR1 was purified from the supernatants by affinity chromatography using a HiTrap Heparin HP column (GE Healthcare, Buckinghamshire, UK), and the eluent was further purified with a centrifugal filter device (Amicon Ultra -15, 100 kDa; Milipore Corporation, MA, USA). The sCR1 concentration was determined using a micro BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

For thiolation, 1 mL of 3 mg/mL sCR1 solution was mixed with 100 μL of 10 mg/mL Traut's reagent in phosphate-buffered saline (PBS; MP Biomedicals, Illkirch, France). The solution was left at room temperature for 1 hour, after which thiolated sCR1 (sCR1-SH) was purified by a Sephadex G25 column (GE Healthcare). Ellman's reagent (Nacalai Tesque, Kyoto, Japan) was used to determine the number of thiol groups per sCR1 molecule.

2.2 Surface plasmon resonance (SPR) monitoring of sCR1-heparin interactions

The interaction between sCR1 and heparin was monitored by an in house-designed SPR instrument [25], with the SPR sensor surface consisting of a 1-nm chromium layer and a 49-nm gold layer on a BK-7 glass plate (refractive index 1.515; Arteglass Associates Co., Kyoto, Japan). A self-assembled monolayer (SAM) of 1-dodecanethiol (Wako Pure Chemical, Osaka, Japan). A hydrophobic monolayer (CH_3-SAM) was formed on the gold-coated glass by incubation in 10 mM 1-dodecanethiol in ethanol at room temperature overnight. The glass plate was set on a prism of the SPR apparatus and the flow cell was assembled on the glass plate. The intensity of the reflected light was monitored in real-time when each solution was applied to the flow cell. Maleimide-poly(ethylene glycol)-conjugated phospholipid (Mal-PEG-DPPE) was synthesized as described previously [20]. After Mal-PEG-DPPE solution (100 μg/mL in PBS) was applied to the CH_3-SAM surface, sCR1-SH (100 μg/mL in PBS) was applied for 30 min at 37 °C. After washing with PBS, heparin (1 mg/mL in PBS) and sCR1 solutions (100 μg/mL in PBS) were alternately flowed for 30 min at 37 °C for each step.

2.3 Immobilization of sCR1 and heparin on the islet surface

Pancreases from ACI/N rats (8-week-old males; Shimizu Co., Japan) were digested by the collagenase method and islets were purified from the digested tissue using a discontinuous density gradient of Ficoll/Conray solutions [26]. Islets were maintained in culture medium (RPMI-1640; Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin for 5 days.

sCR1 and heparin were immobilized on the islet surface as illustrated in Scheme 1. Islets were incubated in Mal-PEG-DPPE solution (1 mg/mL in PBS) for 20 min at room

temperature to prepare the Mal-PEG-islets [20]. After washing with PBS, Mal-PEG-islets were incubated in a solution of sCR1-SH (1 mg/mL in PBS) for 1 hour at room temperature to allow sCR1 immobilization on the islet surface (sCR1-islets). sCR1-islets were washed three times with serum-free medium M199 and incubated in a heparin solution (5 mg/mL in medium M199) for 30 min at room temperature to prepare the first double-layer (sCR1-heparin) on the islet surface. Subsequent sCR1-heparin layers were deposited onto the first layer by incubating sCR1-heparin-islets in sCR1 (500 μg/mL in M199) and heparin (5 mg/mL in M199) alternately for 30 min at room temperature until the desired number of layers was achieved. Finally, the islets were washed with culture medium.

2.4 Observation of sCR1 and heparin immobilized on islet surfaces

Immunostaining of immobilized sCR1 was carried out using anti-sCR1 J3D3 antibodies (Invitrogen) as primary antibody and fluorescent-Alexa488 labeled anti-mouse antibody (Beckman Coulter, USA) as counterstain. Fluorescein isothiocyanate (FITC)-conjugated sCR1 (FITC-sCR1) was prepared for islet observation. Briefly, 1 mL of sCR1 solution (3 mg/mL) was mixed with 100 μL FITC (0.12 mg in 50 μL dimethyl sulfoxide; Dojindo Laboratories, Kumamoto, Japan) at a molar ratio of 1:20. The mixture was agitated at room temperature for 6 hours. FITC-sCR1 was separated from un-reacted reagent using a Sephadex G25 column and stored in the dark at 4 °C until use. Stained islets were observed with confocal laser scanning microscopy (FLUOVIEW FV500, Olympus, Tokyo, Japan).

FITC-heparin was prepared as described previously [13]. One milliliter of heparin (40 mg/mL in pure water) was mixed with FITC (2.6 mg in 100 μL dimethyl sulfoxide) and the mixture was left at room temperature overnight. The crude product was precipitated in an 8:2 mixture of acetone and diethylether, evaporated, and washed three times with acetone. The precipitate was then dialyzed against PBS for 2 days using dialysis cassettes (3500 Da; Thermo Scientific) to obtain FITC-heparin.

2.5 Anti-thrombin activity of immobilized sCR1-heparin

To evaluate the anti-thrombin activity of sCR1-heparin-immobilized substrate surfaces, a silicon sheet (10 mm thick) with a hole (6 mm diameter, 4 wells) was placed on a CH_3-SAM glass plate. One hundred microliters of Mal-PEG-DPPE solution (500 μg/mL in PBS) was applied to each well and left for 30 min at room temperature. The wells were washed five times with PBS. One hundred microliters of sCR1-SH (100 μg/mL in PBS) was applied to each well and left for 1 hour at room temperature. After washing with PBS, heparin (1 mg/mL in PBS) and sCR1 solution (100 μg/mL in PBS) were sequentially applied and left for 30 min after each step until the desired number of sCR1-heparin layers was formed. As a control, a solution of cystein (1 mM in PBS) was added to the Mal-PEG-DPPE-immobilized wells and left for 1 hour.

Anti-thrombin activity was examined via enzyme-linked immunosorbent assay (ELISA) using the Sensolyte® 520 thrombin activity assay kit (AnaSpec, CA, USA) with a slight modification. Tris-HCl buffer (100 μL) with or without antithrombin III (10 μg/mL) and 50 μL thrombin solution (1 μg/mL in assay buffer) was sequentially added to each well and the plate was incubated for 10 min at 37 °C. Fifty microliters of the

thrombin-substrate solution were then added to each well. The reaction mixtures were incubated for 30 min at 37 °C and the fluorescence (excitation 490 nm, emission 520 nm) was measured by a fluorophotometer (F-2500; Hitachi, Co., Tokyo, Japan).

2.6 Protective effect of sCR1 from complement-mediated cytotoxicity

The protective effect of immobilized sCR1 from antibody/complement-mediated cytotoxicity was examined as described previously [19, 20]. Blood was drawn from a New Zealand white rabbit (20-week-old male; Shimizu Co.), applied to glass test tubes, and allowed to clot for 30 min at room temperature. The tubes were centrifuged at 2000 rpm at 4 °C for 30 min. Serum was collected as the supernatant, pooled, filtered through a 200-nm membrane filter, and stored at -80 °C until use. The complement in the rabbit serum was inactivated by heating the serum at 56 °C for 30 min. Twenty islets from each group were incubated in 1 mL of 50% rabbit serum at 37 °C. Serum was exchanged every 24 hours. Islet morphology was observed every 24 hours under a phase contrast microscope (IX71, Olympus Optical Co. Ltd., Tokyo, Japan). Insulin levels in the collected sera were determined by ELISA (Shibayagi, Gunma, Japan).

2.7 Insulin secretion by modified islets

A static glucose stimulation assay was carried out to determine whether insulin secretion function deteriorates following immobilization of sCR1 and heparin [27]. Fifty islets were sequentially incubated in Krebs-Ringer buffer containing 0.1 g/dL or 0.3 g/dL glucose, with each incubation proceeding for 1 hour at 37 °C. Supernatants were collected and insulin concentrations were determined by ELISA.

3. Results

3.1 SPR monitoring of sCR1-heparin interactions

Interactions between sCR1 and heparin on a gold-coated substrate were monitored by an SPR apparatus (Fig. 1A). When Mal-PEG-DPPE was applied to the CH_3-SAM surface, an increase in SPR signal was observed (Fig. 1A), reflecting the immobilization of Mal-PEG-DPPE through the hydrophobic interaction between the alkyl chains of DPPE and CH_3-SAM [20]. When sCR1-SH with an average of 10.8 thiol groups per molecule was applied, (Fig. 1A, first arrow), the SPR signal increased, demonstrating binding of sCR1 to the maleimide group of Mal-PEG-DPPE. The SPR signal underwent step-by-step increases following each application of heparin or sCR1 solutions (arrows, Fig. 1A).

The amounts of sCR1 immobilized after the formation of each double-layer of sCR1 and heparin were determined by the BCA assay (Fig. 1B). The amounts of sCR1 increased with an increase in the number of double-layers (Fig. 1B), an observation consistent with the SPR shifts in Fig. 1A. This result demonstrates the formation of multiple layers of sCR1 and heparin on the CH_3-SAM surface.

3.2 Immobilization of sCR1 and heparin on islet surfaces

Immobilized sCR1 and heparin layers were introduced layer-by-layer to islet cell surfaces (Scheme 1). Cells were treated with Mal-PEG-DPPE to introduce maleimide groups on the surface [20], and sCR1-SH was immobilized on the Mal-PEG-treated islet surface by the reaction between thiol and maleimide. Heparin and sCR1 were alternately added to the surface to form multiple layers. Immunostaining of sCR1 revealed the

presence of sCR1 on the islets, as indicated by fluorescence at the islet peripheries (Fig. 2, panel A1). No fluorescence was observed on islets treated with sCR1 without thiolation (Fig. 2, panel A2). As shown in Fig. 2 (panels B1 and B2), FITC-heparin fluorescence was observed on the surface of sCR1-islets (Fig. 2, panel B1) but no clear fluorescence was detected on naïve islet surfaces (Fig. 2, panel B2), indicating that heparin was immobilized via the interaction with sCR1 molecules on the islets.

We used FITC-sCR1 to examine the stability of multiple sCR1-heparin layers on the islets (Fig. 3). The fluorescence intensity increased as the number of layers increased, indicating that more sCR1 could be immobilized by increasing the number of layers on the islets. Immobilized sCR1 gradually disappeared from islet surface in all cases over time (Fig. 3). As expected, the retention time of sCR1 increased when the number of layers increased; islets with three sCR1-heparin layers maintained intact morphology after three days of culture, indicating that the layer-by-layer method did not influence islet viability.

3.3 Anti-thrombin activity of immobilized heparin

The anti-thrombin activity of heparin immobilized on glass plates (heparin composed the outermost layer) by the layer-by-layer method was determined (Fig. 4). Anti-thrombin activity was detected neither on PEG-treated substrate nor on the sCR1-PEG surface. For the substrate surfaces treated with one or three sCR1-heparin layers, however, the anti-thrombin activity was significantly increased. The immobilized heparin was able to interact with anti-thrombin and activate it to inhibit thrombin. No substantial differences in heparin activity were observed between surfaces with one or three sCR1-

heparin layers just after preparation (Fig. 4). The activity of heparin in one double-layer gradually decreased with time (Fig. 4), reflecting the detachment of heparin from the surface. However, no substantial decreases in activity were observed for three-layer constructs (Fig. 4), suggesting that the layer-by-layer method improves heparin stability and provides an anti-thrombin effect for at least eight days.

3.4 Protective effect of immobilized sCR1 from complement-mediated cytotoxicity

We exploited the fact that rabbit serum contains preformed antibodies against rat antigens to examine complement-mediated cytotoxicity in our system. Islets, sCR1-islets, sCR1-heparin-islets, and islets with three sCR1-heparin layers ((sCR1-heparin)^3-islets) were incubated in 50% rabbit serum; the outermost layer of sCR1-heparin-islets and (sCR1/heparin)^3-islets consisted of heparin. The morphologies of these islets were observed over time via phase contrast microscopy (Fig. 5A). Naïve islets were rapidly destroyed; a number of swelled cells were already detectable after 1 hour of incubation in 50% rabbit serum, and more than half of the cells had swelled after 24 hours of incubation (Fig. 5A). Most islets were completely destroyed within 48 hours (Fig. 5A). When we incubated islets in 50% heat-inactivated rabbit serum, inactivating the complement system, no islet damage was observed after 24 hours of incubation (Fig. 5A, panels a and b). sCR1-islets, sCR1-heparin-islets, and (sCR1-heparin)^3-islets maintained their morphologies for a longer time than naïve islets (Fig. 5A). After 48 hrs, a small number of damaged cells were found at the periphery of the islets.

When naïve islets were incubated in 50% rabbit serum for 24 hours, a large amount of insulin (498.8 ng/20 islets) was found in the supernatant (Fig. 5B). During the

second 24-hour period, however, the amount of leaked insulin decreased, since only a small number of viable islets remained (Fig. 5B). When islets were incubated in 50% heat-inactivated rabbit serum, we detected 6.45 ng of insulin from 20 islets, an amount comparable to the physiological secretion of insulin by islets. During the first 24-hour incubation in 50% rabbit serum, the amounts of insulin leaked from sCR1-islets, sCR1-heparin-islets, and (sCR1-heparin)[3]-islets were 4.3 ng, 7.2 ng, and 56.8 ng per 20 islets, respectively, with the sCR1-islet and sCR1-heparin-islet values at the same level as that of naïve islets incubated in heat-inactivated serum. Thus, the islets were effectively protected from antibody/complement-mediated cytotoxicity by the immobilized sCR1. A relatively high insulin leakage was observed in (sCR1-heparin)[3]-islets due to the slight damage of these cells. Substantial insulin leakages were observed in sCR1-islets, sCR1-heparin-islets, and (sCR1-heparin)[3]-islets during a third 24-hour incubation (Fig. 5B). However, insulin leakage decreased with increasing durations of sCR1 immobilization on the islets; the protective effect of sCR1 was observed more clearly with (sCR1-heparin)[3]-islets than with sCR1-heparin-islets (Fig. 5B).

3.5 Glucose-stimulated insulin secretion

Finally, we examined the effects of sCR1 and heparin immobilization on insulin release (Fig. 6). As the glucose concentration in Krebs-Ringer buffer increased from 0.1 g/dL to 0.3 g/dL, the insulin secretion of naïve islets increased 17.7-fold. Similar insulin release behaviors were observed one-layer through five-layer sCR1-heparin-islets (Fig. 6). No substantial differences were observed between any groups of islets.

4. Discussion

In the present study, we modified sCR1 to carry thiol groups and immobilized it to islets via maleimide-PEG-lipid (Scheme 1). Heparin was co-immobilized on the islets with sCR1 by simple addition of a heparin solution to the islet suspension, because sCR1 has a strong affinity for heparin [24, 28, 29]. After formation of the first double-layer of sCR1-heparin, additional double-layers were easily formed layer-by-layer by simply repeatedly alternating the solutions. Previously, heparin was immobilized onto islet surfaces via avidin, leading to the evasion of IBMIR [10]. Although effective, this method is hardly applicable to human patients because avidin is a xenogeneic protein isolated from chicken eggs [30] and thus may cause unfavorable immune reactions.

We aimed to inhibit blood coagulation and complement activation to prevent early islet graft loss caused by IBMIR. Heparin and sCR1, a potent inhibitor of the classical and alternative complement activation pathways, were alternately immobilized on islets (Scheme 1). Although heparin is known to lose anti-thrombin activity when complexed with molecules such as protamine [31], our system demonstrated anti-thrombin activity (Fig. 4) and protective effects from antibody/complement cytotoxicity (Fig. 5) regardless of the number of sCR1-heparin layers formed on the islets.

There is no consensus on the period required to inhibit blood coagulation and complement activation to prevent early islet graft loss. We aimed to increase the retention period of heparin and sCR1 on islets with increasing numbers of sCR1-heparin double-layers. Heparin in three double-layers exhibited stronger anti-thrombin activity than that in one double-layer after 8 days of culture (Fig. 4). sCR1 immunostaining, however, did not reveal a clear relationship between sCR1 retention period and the number of double-

layers (Fig. 3). sCR1, however, was clearly seen on the islets regardless of the number of double-layers, and the protective effect of immobilized sCR1 was detectable after two days of culture.

The first two days following transplantation are the most severe for islets due to IBMIR, specifically the activation of the coagulation system and the complement cascade [2, 3]. A large quantity of islets was destroyed in this period. Although immobilized sCR1 and heparin were gradually released from the cell surface over several days, our approach will provide beneficial effects for clinical islet transplantation.

5. Conclusions

sCR1 and heparin can be alternately immobilized on islet surfaces using a mild and simple method that does not deteriorate islet cell viability or function. Anti-complement activation and anti-coagulation activities were also retained in this system, which could be employed to reduce the number of islets required to reverse hyperglycemia and prolong graft survival in both allo- and xeno-islet transplantation.

6. Acknowledgements

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Scheme and Figure Captions

Scheme 1. Immobilization of sCR1and heparin on the islet cell surface.

Figure 1. sCR1 and heparin layer formation via affinity-based interactions. (A) Monitoring of sCR1 and heparin layer formation by SPR. A Mal-PEG-DPPE solution was applied to CH_3-SAM on a gold-coated glass plate, sCR1-SH was applied, and heparin and sCR1 were sequentially applied to the surface. (B) The amounts of sCR1 in the layers was determined by BCA assay. (sCR1 and heparin) is one double-layer (DL).

Figure 2. Immobilization of sCR1 and heparin on islet surfaces. (A) Immunostaining of sCR1 on sCR1-islets (A1) and naive islets (A2). (B) sCR1-islets (B1) and PEG-islets (B2) were exposed to FITC-heparin. All islets were observed via confocal laser scanning microscopy. (C) Phase contrast image of islets immobilized with three sCR1-heparin double-layers after 3 days of culture. Scale bar: 200 μm.
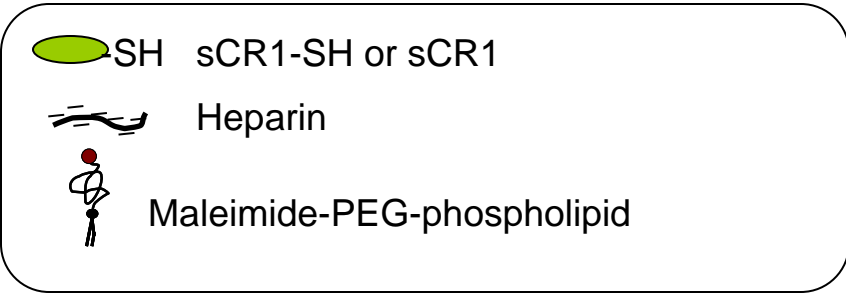
Figure 3. Retention of sCR1 on islet surfaces. Multiple sCR1-heparin layers were formed by sequential exposure of sCR1-islets to heparin and sCR1 solutions (1L = one layer, 2L = two layers, and so on). FITC-sCR1 was used to visualize immobilized sCR1. Islets with multiple layers of sCR1 and heparin were observed by confocal laser microscopy after the islets were maintained in culture medium for the indicated periods. Scale bar: 200 μm.

Figure 4. Relative thrombin inactivation activities of multiple sCR1-heparin layers on glass plates. The activities were determined after the glass plates were maintained in culture medium (medium RPMI-1640 containing 10% FBS) for the indicated periods.

Figure 5. Protective effects of sCR1 from antibody/complement cytotoxicity. (A) Phase contrast microscopy of sCR1-islets, sCR1-heparin-islets, (sCR1-heparin)[3]–islets, and naïve islets maintained in 50% rabbit serum for the indicated periods. Images of islet morphology were taken before (a) or after (b) incubation in 50% heat-inactivated rabbit serum. (B) Amounts of insulin leakage from the islets into 50% rabbit serum every 24 hours.

Figure 6. Static glucose stimulation of naïve islets, sCR1-islets, and islets immobilized with one, three, or five double-layers of sCR1-heparin. Insulin concentrations were determined by ELISA.

Scheme 1

SH   sCR1-SH or sCR1

Heparin

Maleimide-PEG-phospholipid

Inhibition of complement activation
and coagulation reaction

Hydrophobic
interaction

Thiol-maleimide
reaction

Layer-by -layer

Cell membrane of islet

Figure 1

(A)

(B)

Figure 2

Figure 3

	sCR1-1L	sCR1-2L	sCR1-3L	sCR1-4L	sCR1-5L
Day 0					
Day 1					
Day 2					
Day 3					

Figure 4

Figure 5(A)

(A)

	sCR1- islets	sCR1-Heparin-islets	(sCR1-Heparin)^3 -islets	Naive islets
0h				
24h				
48h				
72h				

(a)   (b)

Figure 5(B)

(B)

Figure 6